THE COPPER CONTENT AND THE MINIMAL MOLECULAR WEIGHT OF THE HEMOCYANINS OF *BUSYCON CANALICULATUM* AND OF *LOLIGO PEALEI*

HUGH MONTGOMERY

(*From the Department of Physiology, Harvard Medical School, Boston, and the Marine Biological Laboratory, Woods Hole*)

The view, originally put forward by Fredericq (1878), that copper is a normal constituent of hemocyanin and that it has a significance in the respiratory function of this protein similar to that of iron in hemoglobin has been substantiated by later investigations, particularly those of Begemann (1924) and Redfield, Coolidge and Montgomery (1928), which show that the combining ratio of copper to oxygen is the same in the blood of a large number of invertebrates. A knowledge of the quantity of copper in hemocyanin consequently provides significant information with regard to its respiratory function. Inasmuch as the amount of copper in the various hemocyanins does not appear to be the same, such data gives unequivocal evidence of the specific character of the respiratory pigments in the different groups of invertebrates. Furthermore, because of the very small number of copper atoms in the hemocyanin molecule, the copper content is a most valuable basis from which to estimate the minimal molecular weights of these proteins.

In this paper an investigation of the hemocyanin of the whelk, *Busycon canaliculatum*, and of the squid, *Loligo pealei*, is described. Mendel and Bradley (1906) studied the respiratory protein of the blood of the whelk, which they called hemosycotypin,—a name derived from the then current generic name of this form, *Sycotypus*. They report that it contained zinc as well as copper.[1] They concluded that copper composed only 0.043 per cent of the weight of the molecule, a value very much smaller than that obtained in the case of other hemocyanins and one which leads to very high estimates of the protein content of the blood when the oxygen capacities demonstrated by

[1] It seems preferable to include "hemosycotypin" among the hemocyanins because it has been demonstrated that the combining ratios of copper and oxygen are the same in this case as in that of other hemocyanins and because recent observations in this laboratory appear to make it doubtful whether the zinc is a true constituent of the protein molecule. Inasmuch as specific differences appear to exist between the hemocyanins of different groups of animals, confusion will be apt to result if each hemocyanin is given a different specific name.

Redfield, Coolidge and Montgomery (1928) are taken into account. The copper content of the hemocyanin of the squid does not appear to have been previously examined.

The copper content of these hemocyanins has been determined on material purified according to several standard procedures applicable to protein substances. Analyses for copper were made by the method described by Redfield, Coolidge and Shotts (1928). Between 10 and 20 c.c. of the hemocyanin solutions were used in each sample. The samples were dried in an oven at 100–110° C. for 48 hours, cooled in a dessicator and weighed. This procedure was repeated daily until successive weights did not vary more than 1 mgm. The samples of dried hemocyanin weighed between 100 and 300 mgm. Digestion, the electrolytic separation of copper, and its estimation were carried out exactly as described, except that in the titration 15 drops of potassium iodide were used instead of 10, as this modification was found to sharpen the end point.

We have not succeeded in producing definitely crystalline preparations of the hemocyanin of *Busycon canaliculatum* by methods which have been found applicable in other cases. Dhéré, Baumeler and Schneider (1929) have also been unsuccessful in crystallizing this hemocyanin. However, on prolonged dialysis against distilled water a precipitate is formed which appears to be composed of short rods and which gives a silky sheen on shaking similar to that characteristic of crystalline protein preparations.[2] *Busycon* hemocyanin appears to be a globulin, as it is insoluble in the region of its isoelectric point in salt solutions of sufficient dilution. This property has been used in purifying our material as well as the usual procedure of salting out with ammonium sulphate, employed by Redfield, Coolidge and Shotts (1928) in the preparation of *Limulus* hemocyanin.

[2] In an attempt to produce crystals, a number of preparations of hemocyanin, all of which showed a silky sheen on shaking, have been made by different methods from several species. The precipitated particles were too small, however, to be recognized under the microscope as definite crystals, though a very fine rod shape was observed in many cases. By the addition of 2 drops of serum to 1–2.5 c.c. of 0.05M acetate buffer solution of pH 4 to pH 5, the hemocyanins of *Busycon canaliculatum* and of *Busycon carica* were precipitated and showed a sheen on shaking. In the case of the bloods of the eight different species; *Limulus polyphemus* (horseshoe crab), *Busycon canaliculatum*, *Busycon carica*, *Libinia emarginata* (spider crab), *Loligo pealei*, *Homarus americanus* (lobster), *Callinectes sapidus* (blue crab), and *Ovalipes ocellatus* (lady crab), the hemocyanin was precipitated by diluting the serum 20 to 200 times and adding a few drops of 0.006 per cent acetic acid to 5 c.c. of the diluted serum. The acid must be added slowly or a precipitate will be formed which will show no sheen. Too much acid redissolves the precipitate.

In several cases these hemocyanin precipitates were concentrated by centrifuging and redissolved, whereupon the solutions appeared distinctly blue. This color disappeared when the solution was reduced with sodium hydrosulfite so that evidently the hemocyanin was not denatured by the process.

TABLE I

Copper Content of Hemocyanin of Busycon canaliculatum

Specimen No.	Method of Preparation	Dry Weight	Copper	Copper
		grams	*mgm.*	*per cent*
IVa	Three washings at isoelectric point	0.2071	0.496	0.240
		0.2053	0.496	0.242
		0.2067	0.492	0.238
		0.2070	0.482	0.234
IVb	Four additional washings at isoelectric point	0.1541	0.366	0.237
		0.1538	0.378	0.245
		0.1544	0.371	0.240
		0.1554	0.378	0.243
VI	Three washings at isoelectric point	0.0914	0.225	0.246
		0.0919	0.204	(0.227)
		0.0916	0.217	0.238
		0.0917	0.230	0.238
		0.2694	0.642	0.238
		0.2699	0.634	0.235
		0.2698	0.633	0.235
		0.1816	0.437	0.241
		0.1821	0.433	0.239
VII	Salting out and dialysis	0.1694	0.440	0.260
		0.1680	0.436	0.260
		0.1700	0.441	0.260
		0.1699	0.443	0.260
		0.1693	0.436	0.258
		0.1693	0.438	0.258
		0.1693	0.434	0.256
		0.1693	0.440	0.260
		0.1693	0.441	0.260
		0.1693	0.434	0.256
VIII	Salting out and dialysis under conditions leading to precipitation	0.1075	0.263	0.242
		0.2130	0.517	0.242
		0.2120	0.530	0.250
X	Salting out and dialysis	0.3967	0.948	0.239
		0.3948	0.944	0.238
		0.3965	0.950	0.238
		0.3978	0.948	0.237
		0.6492	1.535	0.236
		0.6485	1.554	0.240
		0.6487	1.534	0.236
XI	Salting out and dialysis	0.5494	1.318	0.240
		0.5481	1.308	0.238
		0.5483	1.309	0.239
		0.5478	1.311	0.239
		0.5472	1.315	0.240

Specimen IVa was made from blood which had been preserved with toluene in the cold room for two weeks. It was diluted with ten times its volume with distilled water and brought into the region of its isoelectric point by the careful addition of 0.01N HCl. The precipitate resulting was separated by centrifuging and put into solution in the original volume of water by the addition of an amount of sodium hydroxide equivalent to the hydrochloric acid previously added. This process was twice repeated. The precipitate finally obtained was washed with distilled water. The final product contained only a trace of chloride. Whenever acid or alkali was added, it was run in through a glass tube which had been drawn to a fine point while the hemocyanin was being vigorously stirred. In order to determine whether further purification of this product could be obtained, the entire process of purification was repeated four more times on a portion of Specimen IVa, the resulting preparation being designated Specimen IVb. Specimen VI was made in a manner similar to Specimen IVa. Specimen VII was made from blood which had been preserved half-saturated with ammonium sulphate for a month. The precipitated hemocyanin was separated by centrifuging and dissolved in a large volume of 5 per cent saturated solution of ammonium sulphate. The solution was centrifuged in order that a small amount of insoluble material might be discarded, and the solution was reprecipitated by the addition of saturated ammonium sulphate. This process was repeated twice. The solution was then dialyzed against 0.001N sodium hydroxide under 20 cm. Hg reduced pressure for two weeks, at the end of which time it was free of sulphate. The preparation of Specimen VIII included the same steps as Specimen VII, except that it was dialyzed against 0.001N sodium hydroxide for five weeks at atmospheric pressure. At the end of the fifth week a precipitate appeared in the solution which gave on shaking a silky sheen similar in appearance to that produced by protein crystals. The precipitate consisted of rod-shaped particles about 2 μ in length. The solution still contained traces of sulphate and was consequently centrifuged and the precipitate washed three times with a large volume of distilled water. The sulphate test was then negative. Specimens X and XI were prepared from material which had been kept over two years precipitated in half saturated ammonium sulphate. They were purified by reprecipitation with ammonium sulphate (pH 8.0), repeated three times, followed by dialysis against 0.0001 sodium hydroxide for 18 days. The preparation and analysis of Specimens X and XI were made by Miss Elizabeth Ingalls.

The results of the analyses of these preparations are given in

Table I. The copper content obtained in the case of preparations made in the various ways is very nearly the same. This fact may be taken as evidence that fairly pure preparations of the protein have been obtained. The fact that the copper content of Specimen IVb was not materially increased over that in Specimen IVa by additional washing is further evidence for the adequacy of the method of purification employed.

The best representative value of the copper content of *Busycon canaliculatum* hemocyanin appears to be 0.24 per cent. Specimen VII yields consistent values 0.02 per cent higher than this. Inasmuch as Specimens VIII, X and XI, prepared by the same general method, agree with the general series, it is probable that the high value obtained in the case of Specimen VII should be attributed to some systematic analytical error rather than to superiority in the method of preparation.

Two specimens, which were obtained by the dialysis of fresh blood without other attempt at purification, yielded a product which contained about 0.22 per cent copper. This material was free of chloride and had the same nitrogen content per unit weight as the others. The result would appear to indicate that another protein may be present in the blood, but that if so, it exists only in small amounts. In the case of *Limulus*, the hemocyanin appears to account for about 95 per cent of the protein of the serum. In order to investigate this possibility further an attempt has been made to determine how far the nitrogen content of the blood of *Busycon canaliculatum* may be accounted for by the hemocyanin contained in it as estimated from the quantity of copper present. The nitrogen content of Specimen X was determined by the Kjeldahl method. Successive analyses yielded 15.6; 15.5; 15.7; 15.5; 15.4; 15.7; mean 15.5 grams nitrogen per 100 grams dry weight. The copper content of Specimen X was 0.238 grams per 100 grams dry weight. One part of copper consequently corresponds to 65.2 parts of nitrogen. Two specimens of blood were analyzed for copper and nitrogen. The first contained 0.074 mgm. copper per c.c. and 4.92 mgm. nitrogen per c.c. From the copper content it may be estimated that it contained 4.84 mgm. nitrogen as hemocyanin. The second specimen of blood contained 0.066 mgm. copper per c.c. and 4.14 mgm. nitrogen per c.c. The hemocyanin concentration as estimated from the copper content would account for 4.3 mgm. nitrogen. It is evident from these measurements that hemocyanin will account approximately for all of the protein nitrogen in *Busycon* blood.

One preparation of the hemocyanin of the allied species, *Busycon*

carica, was made. The blood had been preserved in a precipitated condition in half-saturated ammonium sulphate for one year in the cold room. The hemocyanin was separated, purified by the procedure employed in the case of *Busycon canaliculatum* Specimen X. Analysis of the copper content of the purified material yielded the following values: 0.217, 0.235, 0.238 per cent. The copper content of the hemocyanin of this species appears to be approximately the same as that of *Busycon canaliculatum*.

The hemocyanin of the squid, *Loligo pealei*, may be readily crystallized by methods similar to those first employed by Henze (1901) in preparing crystalline *Octopus* hemocyanin, and consequently lends itself well to purification. Squid hemocyanin is insoluble in solutions containing high concentrations of ammonium sulphate. It was found that if enough saturated ammonium sulphate solution is added to the blood to form a very slight cloud of precipitated hemocyanin, a fuller precipitation in the form of crystals can then be produced by several procedures designed to decrease the solubility of the hemocyanin in the solution. These were: (1) the careful addition of increasing quantities of ammonium sulphate, (2) increasing the hydrogen ion concentration as in the Hopkins-Pinkus (1898) method of crystallizing albumen, or (3) raising the temperature. These methods can be used with success in combination. Crystallization by raising the temperature, which is presumably due to increasing the "salting out" effect of the ammonium sulphate at the higher temperature is particularly efficacious and has the advantage that it involves the addition of no reagents and may consequently be accomplished slowly so as to favor the formation of crystals. It was found that by raising the temperature from 0° C. to 30° C., a heavier crystalline precipitate is produced than by raising it to room temperature only. A temperature change within a range which will not denature the protein did not crystallize all the hemocyanin that was in the solution. Consequently, the yield may be increased by combining the temperature method with the addition of ammonium sulphate or of acid. When crystallization is produced in this manner, there is formed first a fine precipitate, visible under the microscope but apparently amorphous. This changes in a few minutes to fine rods and then to bundles of needles and finally to large needles. The process is much like that described in the case of *Eledone moschata* hemocyanin by Kobert (1903). The appearance of the crystalline rods is similar to that figured by Dhéré (1919, figure 4), in the case of the oxyhemocyanin of *Helix pomatia* formed in the presence of sodium sulphate. If large excess of reagents are added suddenly, the precipitate produced is

amorphous. Crystallization of squid hemocyanin was obtained more readily from fresh blood than from preparations which had been preserved in a precipitated condition in concentrated ammonium sulphate or from previously crystallized hemocyanin. Crystals which had been kept for a year in the cold room in their mother liquor (half saturated ammonium sulphate), were found to have become insoluble in distilled water. This phenomenon was observed by Craifaleanu (1919) in the case of crystals of the hemocyanin of *Octopus vulgaris*. Craifaleanu called this form "para-hemocyanin."

TABLE II

Copper Content of Hemocyanin of Loligo pealei

Specimen No.	Method of Preparation	Dry Weight	Copper	Copper
		grams	*mgm.*	*per cent*
I	Salting out and dialysis	0.1485	0.384	0.258
		0.1486	0.371	0.250
		0.1502	0.388	0.257
		0.1490	0.376	0.252
II	Crystallization and dialysis	0.0785	0.194	0.244
		0.1620	0.386	0.238
		0.1624	0.390	0.242
V	Salting out and dialysis	0.4579	1.155	0.252
		0.4594	1.161	0.254
		0.4593	1.178	0.256
		0.4601	1.159	0.252
		0.4592	1.154	0.252

Analyses of the copper content of the hemocyanin of *Loligo pealei* have been made upon three preparations. Specimens I and V were prepared from blood which had been precipitated by the addition of ammonium sulphate to half saturation and kept in the cold room at about 5° C. for two years. The material had a fishy odor, which disappeared when it was shaken with air and from which the final preparations were entirely free. The precipitate was separated from the supernatant fluid with the centrifuge and was dissolved with a small volume of 5 per cent ammonium sulphate. The solution was again centrifuged to throw down any insoluble material, and the fluid was drawn off and reprecipitated by the addition of saturated ammonium sulphate. This process was repeated twice. The solution was finally dialyzed until it was found to be free of sulphate. Specimen II was prepared by crystallization from fresh blood. The blood was chilled to 0°, and then sufficient saturated ammonium sulphate was added to

produce a very slight precipitation of hemocyanin. The temperature was then raised from 0° to 20°, when full precipitation was obtained. The precipitate was in the form of needle-shaped crystals about ten μ in length. The crystals were separated from the mother liquor by centrifuging and dissolved with 5 per cent saturated ammonium sulphate. Insoluble material was removed by centrifuging, and the hemocyanin was then reprecipitated as before. This second precipitate was not crystalline, however. The preparation was then dialyzed against water until free of ammonium sulphate. All three preparations had a clear blue-green color and became colorless in the characteristic way upon reduction with sodium hydro-sulphite.

Table II contains the data obtained from analyses of these preparations of squid hemocyanin, which all yield values for the quantity of copper in the molecule close to 0.25 per cent.

It is interesting to compare the values obtained for the copper content of the hemocyanin of *Busycon* and *Loligo* with those previously reported for other species, particularly with regard to their systematic relationships. In Table III are collected the various determinations

TABLE III

	Copper	Author
	per cent	
Cancer	0.32	Griffiths (1892).
Homarus	0.34	"
Sepia	0.34	"
Octopus vulgaris	0.38	Henze (1901).
Loligo pealei	0.25	
Helix pomatia	0.25	Burdel (1922).
" "	0.29	Begemann (1924).
Busycon canaliculatum	0.24	
Limulus polyphemus	0.173	Redfield, Coolidge and Shotts (1928).

of the copper content of hemocyanin which occur in the literature. It is noteworthy that the value obtained in the case of *Busycon canaliculatum* and *Busycon carica* does not differ greatly from those attributed to the other gastropod, *Helix pomatia*. The value obtained for *Helix pomatia* by Begemann, whose method of copper analysis we have employed, exceeds the value obtained with *Busycon* by an amount well in excess of the apparent experimental errors. These hemocyanins appear also to differ in certain other respects. *Busycon* hemocyanin cannot be crystallized by methods which succeed in the case of *Helix* (Dhéré, Baumeler and Schneider, 1929). *Busycon* hemocyanin is insoluble in the region of its isoelectric point in the presence of quite

appreciable amounts of salt. *Helix* hemocyanin, on the other hand, appears to be readily dissolved by very small concentrations of salt under these circumstances (Svedberg and Heyroth, 1929).

It is surprising that such a great difference exists between the copper content of the hemocyanin of the squid and that of the octopus. Inasmuch as the properties of the respiratory pigments in these two cephalopods appear to be very similar, we believe it to be desirable to redetermine these values by methods of preparation and analysis which are strictly comparable.

The weight of hemocyanin containing one atom of copper is given by dividing the atomic weight of copper, 63.57, by the fraction of the weight of hemocyanin due to this element. In the case of *Busycon canaliculatum* this fraction is 0.25×10^{-2}. The minimal molecular weight of *Busycon* hemocyanin thus appears to be approximately 26,500, when estimated upon the basis of its copper content. It has been shown, however, by Redfield, Coolidge and Montgomery (1928), that when hemocyanin becomes associated with oxygen to form oxyhemocyanin, one molecule of oxygen is combined with a quantity of hemocyanin containing two atoms of copper. Inasmuch as it appears highly unlikely that the oxygen molecule is dissociated into its constituent atoms in its reaction with the respiratory protein, it seems safe to assume that each molecule of oxyhemocyanin is combined with not less than one molecule of oxygen. The hemocyanin molecule must consequently contain at least two atoms of copper. Estimated on this basis, the minimal molecular weight of *Busycon* hemocyanin is approximately 53,000. In a similar way it may be calculated that the minimal molecular weight of the hemocyanin of *Loligo pealei*, estimated on the basis of its copper content, is 25,400, and when the oxygen-combining relations are taken into account, the combining weight appears to be approximately 51,000.

Summary

The hemocyanin of *Busycon canaliculatum* contains 0.24 per cent of copper and 15.8 per cent of nitrogen. Its minimal molecular weight is approximately 53,000.

The copper content of the hemocyanin of *Busycon carica* appears to be the same.

The hemocyanin of *Loligo pealei* contains 0.25 per cent of copper and has a minimal molecular weight of approximately 51,000.

REFERENCES

Begemann, H., 1924. Over de ademhalingsfunctie van haemocyanine, thesis, Utrecht; for abstract see Jordan, H., 1925. *Zeitschr. f. vergl. Physiol.*, **2**: 381.

Burdel, A., 1922. Contribution a l'etude des hemocyanines, thesis, Fribourg.

Craifaleanu, A., 1919. *Boll. Soc. Natur. Napoli*, Anno **32**: 88.

Dhéré, C., 1919. *Jour. physiol. et path. gén.*, **18**: 503.

Dhéré, C., Baumeler, C., and Schneider, A., 1929. *Compt. rend. Soc. de biol.*, **101**: 759.

Fredericq, L., 1878. *Arch. de Zool. esp. et gén.*, **7**: 535.

Griffiths, A. B., 1892. *Compt. rend. Acad.*, **114**: 496.

Henze, M., 1901. *Zeitschr. physiol. Chem.*, **33**: 370.

Hopkins, F. G., and Pinkus, S. N., 1898. *Jour. Physiol.*, **23**: 130.

Kobert, R., 1903. *Arch. f. ges. Physiol.*, **98**: 411.

Mendel, L. B., and Bradley, H. C., 1906. *Am. Jour. Physiol.*, **17**: 167.

Redfield, A. C., Coolidge, T., and Montgomery, H., 1928. *Jour. Biol. Chem.*, **76**: 197.

Redfield, A. C., Coolidge, T., and Shotts, M., 1928. *Jour. Biol. Chem.*, **76**: 185.

Svedberg, T., and Heyroth, F. F., 1929. *Jour. Am. Chem. Soc.*, **51**: 539.